

Toxicology Excellence for Risk Assessment



TERA

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best use of toxicity data for risk values*

March 5, 1998

Addressees:

Joe Brown, California EPA, Office of Environmental Health Hazard Assessment
Dan Caldwell, Toxicologist, Belle Meade, NJ
Dorothy Canter, U.S. EPA, Office of Solid Waste and Emergency Response
Charles Capen, Ohio State University, Department of Veterinary Biomedicine
John Christopher, California EPA, Department of Toxic Substances Control
Eric Clegg, U.S. EPA, National Center for Environmental Assessment
Kevin Crofton, U.S. EPA, National Health and Environmental Effects Research Laboratory
Vicki Dellarco, U.S. EPA, Office of Water
Marvin Friedman, Cytec Industries, Inc.
Greg Harvey, U.S. Air Force, Wright-Patterson Air Force Base
Annie Jarabek, U.S. EPA, National Center for Environmental Assessment
Kevin Mayer, U.S. EPA, Region IX
David Morry, California EPA, Office of Environmental Health Hazard Assessment
Mary Jane Selgrade, U.S. EPA, National Health and Environmental Effects Research Laboratory
Marilyn Underwood, California Department of Health Services

Dear Reviewers:

The purpose of this letter is to transmit the protocol for the immunotoxicology study being conducted by the Medical University of South Carolina and to give you a short progress report on the remaining studies in progress. The immunotoxicology protocol was funded by the U.S. Army and reviewed by Mary Jane Selgrade, U.S. EPA. The following documents are attached for your information:

- protocol submitted to the U.S. Army
- comments from Mary Jane Selgrade
- request for protocol amendments in response to comments; memo to MUSC animal care committee
- request for grant modification; letter to Steven Huff, Defense Special Weapons Agency

These documents will be available on the *TERA* homepage shortly.

Progress on the perchlorate studies is continuing. The 90-day and neurobehavioral developmental studies have completed the in-life phase; the hormone analysis and histopathology are in progress. In the 2-generation reproductive study, the parental generation is in the exposure period and the cohabitation period starts March 23. The dose range-finding study in rabbits has been completed; we are awaiting the results of the TSH analysis before making a final selection of doses for the full Segment II study in rabbits. A series of genotoxicity assays including salmonella/mammalian microsome reverse mutation assay, mouse lymphoma assay, and a mouse micronucleus test were started at the end of January.



If you have any questions or comments on the immunotoxicology protocol or any of the studies in progress, you can reach me by phone at (606) 428-2744, or by email at dollarhide@tera.org. As always, thank you for your time and contributions to this project.

Sincerely,

Joan S. Dollarhide
TERA

Enclosures

cc: C. Berrey, U.S. EPA, Region IX
M. Dourson, *TERA*
M. Girard, PSG
D. Mattie, WPAFB
D. Rogers, WPAFB
B. Pohlman, Nevada DEP

From: Dave Mattie <mattied@FALCON.AL.WPAFB.AF.MIL>
To: dollarhide@tera.org <dollarhide@tera.org>
Subject: perchlorate proposal -Forwarded
Date: Thursday, February 19, 1998 4:32 PM

fyi

Received: from RTPMAINHUB-Message_Server by RT-MAIL2.RTP.TOK.EPA.GOV
with Novell_GroupWise; Thu, 06 Nov 1997 17:41:34 -0500
Message-Id: <s462014e.082@RT-MAIL2.RTP.TOK.EPA.GOV>
X-Mailer: Novell GroupWise 4.1
Date: Thu, 06 Nov 1997 17:19:56 -0500
From: MARYJANE SELGRADE <SELGRADE.MARYJANE@EPAMAIL.EPA.GOV>
To: drogers@afsa.jag.af.mil, mattie@FALCON.AL.WPAFB.AF.MIL
Cc: JARABEK.ANNIE@EPAMAIL.EPA.GOV
Subject: perchlorate proposal

At Annie Jarabek's suggestion I am e mailing you this today. I will send a hard copy with attachments via the mail tomorrow.

November 6, 1997

Dr. David R. Mattie
OL AL HSC /OET Bldg.79
2856 G Street
Wright-Patterson AFB, OH 45433-7400

Major Daniel E. Rogers
Air Force Materiel Command Law Office
Environmental Law Directorate
Wright-Patterson AFB, OH 45433

Dear Sirs:

Annie Jarabek asked me to review the "Effects of Ammonium Perchlorate on Thyroid Hormone Levels, Hematopoiesis, and Immune Status of Sprague-Dawley Rats" proposal and respond directly to you. Since I'm an immunotoxicologist I'll confine my comments to the immunotoxicology portion of the proposal.

I think the immune function tests which are proposed to assess immune suppression are reasonable except for T and B cell activation assays listed under thymus. There is no point doing B cell activation in the thymus (there shouldn't be any B cells there); furthermore these two assays (even when done in spleen or lymph node) do not add much to the information that is already being collected from other assays. In addition, these assays tend to have a large amount of variability. Also, I'm wondering if it wouldn't be more rationale to assess phagocytic function with alveolar as opposed to peritoneal macrophages as the former are much more likely to be involved as a first line of defense against invading microbes.

I have big problems with the rationale for doing host resistance models and with the choice of models. The statement on page 4 at the end of paragraph 4, "It is not redundant to include both immunological parameters and host resistance assays in an immunotoxicological study, because changes in immunological parameters may not always reflect changes in resistance to disease (Luster, 1992; 1993)," is problematic for

several reasons. It quotes Luster out of context. The tenor of those two paper was certainly that results of immune function tests are sufficient predictors of enhanced risk of disease. It is certainly EPA's position that the host resistance models are not needed. The outcome of host resistance models depends enormously on the virulence and dose of challenge agent used. In the Luster 1993 paper table 4 shows that the higher the number of tumors in the challenge dose the lower the concentration of immune suppressant needed to see an effect. We showed the same !

thing with mouse cytomegalovirus (Selgrade et al., 1982; Infection and Immunity 38:1046-1055). If this study were to demonstrate effects on immune function, but not on these two infectious agents, you would still have to conclude that there was a risk. If you saw effects on the host resistance model you could never set a safe exposure level based on those results because you could vary that level just by varying the dose of challenge agent. Furthermore, the rats cytomegalovirus model is not well developed. My lab and the Garssen lab are the only two immunotoxicology labs that have worked with it; our results are not identical. The strain of rat used appears to make a difference and no one have done immunotoxicity studies in Sprague-Dawley rats using this virus. I developed the mouse cytomegalovirus model as a host resistance model for immunotoxicity testing and have worked some with the rat model. I would never recommend using the rat model in a routine immunotoxicity s!

tudy of this sort. Even the Garssen study saw little in the way of enhancement except with really extreme immune suppression (whole body irradiation). If you must use a host resistance model the mouse cytomegalovirus model would be a much better bet. In general, the host resistance models in rats are not as well-worked out as their counterparts in mice. I am enclosing a review I wrote on mouse and rat cytomegalovirus as well as a paper on immunotoxicity risk assessment which will soon appear in Toxicology and provides more background on the use of immunotoxicity data in risk assessment. Finally, effects of perchlorate on these two host resistance models will not give you any more information on the potential effects on resistance to tumors than the immune function assays. It's no easier to extrapolated between host resistance models than between immune function data and impact on infectious disease. Hence, unless you are particularly concerned with susceptibility to Listeria or cytomegalovirus, they really don't buy you much.

Some of the effects of ammonium perchlorate, e.g., skin rashes, gastrointestinal irritation, and "hematologic effects," sound like there could be an autoimmune component. It would be far better to try to check this out using an animal model than to proceed with the infectivity models. Methods for testing for autoimmunity have not received the same kind of attention that immune suppression tests have. I would recommend contacting Kimber White at Medical College of Virginia(phone-804-828-6789; FAX 804-828-5604) to ascertain whether any of the models currently being used to test for autoimmunity would be appropriate for the perchlorate assessment. Hypersensitivity is also mentioned. Is perchlorate a contact sensitizer? It might be worth doing a mouse assay for contact sensitivity (either the mouse ear swelling test or the local lymph node assay).

I hope you find this information useful. If you have any questions please give me a call (919-541-1821) or e mail

Sincerely,

MaryJane Selgrade, Ph.D.
Chief, Immunotoxicology Branch
MD 92

**Effects of Ammonium Perchlorate on Thyroid Hormone Levels, Hematopoiesis,
and Immune Status of Sprague-Dawley Rats**

BAA number: DSWA001-BAA01-97

Topic Area: N

Subtopic Area: Environmental Pollutants Research

Date of Quick-Look Submission: March 31, 1997

Date of Quick-Look Return: May 13, 1997

Revised Deadline for Proposal: June 13, 1997

Technical Point of Contact:

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Ammonium perchlorate (AP, NH_4ClO_4) is a white, crystalline solid anion which is used by the Department of Defense (DoD) as an oxidant in solid propellants for rockets and missiles and in munitions. The DoD is interested in the potential toxicity of AP, since its production and storage has resulted in the contamination of soil and water resources on government and contractor installations (TERA, 1996). Many of these polluted sites are being remediated, with the U.S. government ultimately responsible for clean-up cost estimated in the billions of dollars (TERA, 1996). Since the toxicity of AP ultimately determines the extent to which contaminated sites must be remediated, it is in the interest of the DoD to investigate AP's toxicity, rather than to rely on the conservative default assumptions made by regulatory agencies in the absence of sufficient toxicological data.

The disturbance of the thyroid-pituitary axis has long been considered to be the critical effect from exposure to perchlorate salts (EPA, 1995). To date, nearly all of the toxicological investigations of AP have focused on the thyroid gland, the main function of which is to synthesize the circulating hormones, thyroxine (T4) and triiodothyronine (T3). These hormones contain 4 and 3 atoms of iodine, respectively, and are cleaved from a large iodine-containing precursor protein called thyroglobulin (Tg). Among the many tissues containing receptors for the thyroid hormones is the anterior pituitary of the brain. The pituitary generates and releases thyroid-stimulating hormone (TSH) at a rate that is dependent upon circulating levels of T4 and T3. If for any reason circulating levels of T4 and T3 decrease, TSH is released and thyroid function is increased. Thus, thyroid hormone synthesis and secretion is controlled by a sensitive feedback mechanism (Dean and Murray, 1991).

It is clear that AP targets the thyroid gland by virtue of its ability to competitively inhibit the uptake of iodide from the blood by the thyroid gland (Goodman and Van Middlesworth, 1980). This ability is the basis for the use of perchlorate salts medicinally for the control of hyperthyroidism. Since all perchlorate salts dissociate completely when dissolved in body water or aqueous tissues, their toxicities are equivalent. Therefore, the therapeutic use of various perchlorate salts (e.g., potassium perchlorate) can shed light on the toxicological significance of unintentional exposures to AP. All perchlorate salts induce a state of hypothyroidism characterized by a decreased level of serum T4 and T3 and an increased level of TSH (Mannisto *et al.*, 1979; King, 1995). The increased level of TSH overstimulates the thyroid gland leading to a toxic response characterized by an increase in thyroid weight and size, an increase in the number of cells per gland, and ultimately the formation of tumors (Capen, 1992). From a mechanistic standpoint, therefore, it is reasonable to believe that tumor formation will not occur from perchlorate exposures below the level required to produce a decrease in T3 or T4 levels, and a concomitant increase in TSH. For this reason, it is important that the threshold dose for TSH elevation by perchlorate be determined. Although humans with elevated plasma TSH levels are relatively resistant to the development of thyroid cancer versus rats and mice (Hill *et al.*, 1989), the U.S. Environmental Protection Agency (EPA) has classified perchlorate as a B2 or probable human carcinogen (Dollarhide, 1992).

In 1992, a provisional reference dose (RfD) for perchlorate of 1.4×10^{-4} mg/kg/day was derived by the EPA for use in establishing clean-up levels by Superfund personnel (Dollarhide, 1992). A no-observed-adverse-effect-level (NOAEL) of 0.14 mg/kg/day was identified based on a study in which potassium perchlorate was acutely administered to humans to treat the hyperthyroid condition of Graves' disease

(Standbury and Wyngaarden, 1952). In addition, an uncertainty factor of 1000 was judged to be appropriate (i.e., an uncertainty factor of 10 for use of a less than chronic study, 10 for the protection of sensitive individuals, and 10 to account for database deficiencies including the lack of developmental and 2-generation reproduction studies and limited data concerning the possible influence of perchlorate on the hematological system). In 1995, the EPA discussed reducing the uncertainty factor for data base deficiencies from ten to three, and suggested that RfDs in the range of 1 to $5E-4$ mg/kg/day might be appropriate for perchlorate (Dollarhide, 1995). In addition to the human study by Standbury and Wyngaarden (1952), the EPA cited animal studies (Shigan, 1963; Mannisto *et al.*, 1979) to support an RfD in this range. The EPA readily admits that confidence in the provisional perchlorate RfD is low (Dollarhide, 1992).

In response to what it believes to be a provisional RfD that is overly conservative, the Perchlorate Study Group (PSG), a consortium of DoD and industry representatives, proposed to the EPA that a far less conservative provisional RfD be used while additional data on perchlorates were being generated (EPA, 1995). The EPA's response to the PSG's proposal can be summarized as follows: "there are many questions about the chronic effects of perchlorate left unanswered by the existing data. The series of studies that identified a human Frank Effects Level at doses ranging from 6 to 14 mg/kg/day is particularly troubling. Thus, until adequate chronic data are available that addresses the effects of perchlorate on the hematopoietic system, we feel that the appropriate provisional RfD is in the range of 1 to $5E-4$ mg/kg/day" (Dollarhide, 1995). As late as September 1996, the PSG was petitioning the EPA to revise its provisional RfD (TERA, 1996).

The human "frank effects" to which the EPA referred to in its response to the PSG's proposal are those seen following the repeated (i.e., chronic) administration of perchlorate to treat hyperthyroidism. Such effects include isolated reports of skin rashes, sore throat, and gastrointestinal irritation, a fatal case of liver atrophy, and a case of nephrotic syndrome (Environmental Resources Management, Inc., 1995). Of primary concern, however, are the numerous cases of serious hematological effects, several of which have been fatal. The medical literature is replete with case studies of perchlorate-induced agranulocytosis (a disease in which the neutrophil count drops to extremely low levels) (Southwell and Randall, 1960; Sunar, 1963; Barzilai and Sheinfeld, 1966) and aplastic anemia (deficiency in red cell, white cell, and platelet production due to disorders of bone marrow) (Fawcett and Clark, 1961; Hobson, 1961; Krevans *et al.*, 1962).

In contrast to the many human cases, few animal studies have noted effects of perchlorate on immune status or hematopoiesis. Pflugfelder (1959) reported on the failure of the bursa of Fabricius (an organ for the maturation of T lymphocytes) in chickens that had ingested potassium perchlorate. Only two additional studies could be located, the details of which are unavailable, that report perchlorate-induced hematopoietic abnormalities (Voro'yeva, 1969; Selivanova *et al.*, 1973). Surprisingly, there have been no animal studies conducted to address the issue of thyrotoxicity after long-term exposure to low doses of perchlorate. Indeed, only two animal studies employing any exposure regimen have reported a dose-response relationship for perchlorate's effects on the thyroid-pituitary axis (Mannisto *et al.*, 1979; King, 1995). In both of these studies, multiple doses of perchlorate were administered to Sprague-Dawley rats in drinking water, after which serum levels of T3, T4, TSH and Tg were quantitated.

After 4 days of exposure, Mannisto *et al.* (1979) identified a NOAEL level in male rats, based on a decrease in T3 and T4 and a slight increase in TSH levels. More recently, King (1995) has identified NOAELs for AP on TSH levels in male and female rats after 14 days of exposure. An attempt to determine the threshold dose based on hormone level data was made in the latter study, but the author stated that it "appeared that the dose range was not optimum to do so, and that lower doses were needed."

It is clear that data base deficiencies prohibit the derivation of a RfD for perchlorate with any confidence in its accuracy. As noted by the EPA, the data base deficiencies of greatest concern are the lack of developmental and two-generation reproduction studies, the lack of chronic data in general, and limited data concerning the possible influence of perchlorate on the hematological system (EPA, 1995). The Toxicology Division at Wright-Patterson Air Force Base has recently proposed to conduct developmental and multigenerational reproductive toxicity tests of AP (personal communication with Dr. Dave Mattie, WPAFB, OH). To address the remaining issues of exposure duration and hematological effects, we propose to conduct a 90-day exposure study to determine the dose-response functions (including NOAELs and LOAELs (lowest-observed-adverse-effect levels)) for perchlorate's effects on the thyroid, hematopoietic, and immune systems. A single study in which effects on these three systems are examined in the same animals is prudent since significant biological interactions occur between the systems, and all known toxicities of perchlorate to non-thyroid organs may be mediated by thyrotoxicity (EPA, 1995). Consider, for example, that agranulocytosis and aplastic anemia are mediated by the impairment of bone marrow, which in turn is dependent upon T4 for normal hematopoietic development (Wartofsky, 1994). In addition, some of the described hypersensitivities in Graves' disease patients may be attributable to the effects of T4 depletion upon the immune system, especially inhibition of suppressor T cells (Environmental Resources Management, Inc., 1995).

A summary of the proposed toxicological evaluation of rats chronically exposed to AP in drinking water is given in Figure 1 on page 6. Thyroid assessment will include quantitation of T3, T4, TSH, and Tg in serum, and histopathology of thyroid tissue. As previously reported in Sprague-Dawley rats (Mannisto *et al.*, 1979; King, 1995), these parameters are expected to change after exposure to perchlorate. The evaluation of thyroid parameters will not only allow for a comparative analysis with data collected previously using a similar experimental design (King, 1995), but should confirm the absorption of perchlorate in the event that changes are not detected in hematological or immunological parameters. Interestingly, researchers have recently suggested that subtle, diurnally adjusted changes in Tg levels may be the most sensitive index of perchlorate exposure (Environmental Resources Management, Inc., 1995).

Hematological evaluation of the rats will include several red and white cell parameters measured in the peripheral blood and bone marrow (Figure 1). Cellularity and differentials will be performed on the cells obtained from the rat bone marrow, as will stem cell proliferation assays. Thus, any deficiencies in blood cell maturation or effects on mature blood cells will be detected.

Immunological parameters to be measured in this project are also listed in Figure 1. They include descriptive and functional assays, and two host challenge models that are commonly performed in

immunotoxicology studies. Host challenge models include a challenge with *Listeria monocytogenes*, in which resistance is dependent on the cooperation of several immunological parameters such as T cells, natural killer cell, macrophages, and neutrophils (Vos, 1977; Baldridge *et al.*, 1990; Dunn and North, 1991; Czuprynski *et al.*, 1994). The second host resistance model has been developed for immunotoxicity testing specifically in rats and includes a challenge with cytomegalovirus (Garssen, 1995). Resistance to cytomegalovirus is dependent primarily on natural killer cells and cytotoxic T lymphocytes. It is not redundant to include both immunological parameters and host resistance assays in an immunotoxicological study because changes in immunological parameters may not always reflect changes in resistance to disease (Luster, 1992; Luster, 1993). Thus, immunological parameters will assess specific portions of the immune system, while host challenge models will assess the *in vivo* cooperation of several immunological parameters in resistance to bacterial and tumor cell challenge.

The experimental approach described above will enable comparisons to be made between dose-response curves for different toxicological endpoints, information to be gathered on the influence of exposure duration on toxicity, and an examination of the reversibility of perchlorate's effects. The study will address some of the major deficiencies in the perchlorate database, as noted by EPA. The data gathered should lessen the need for conservative default assumptions that drive RfDs downward, and remediation costs upward.

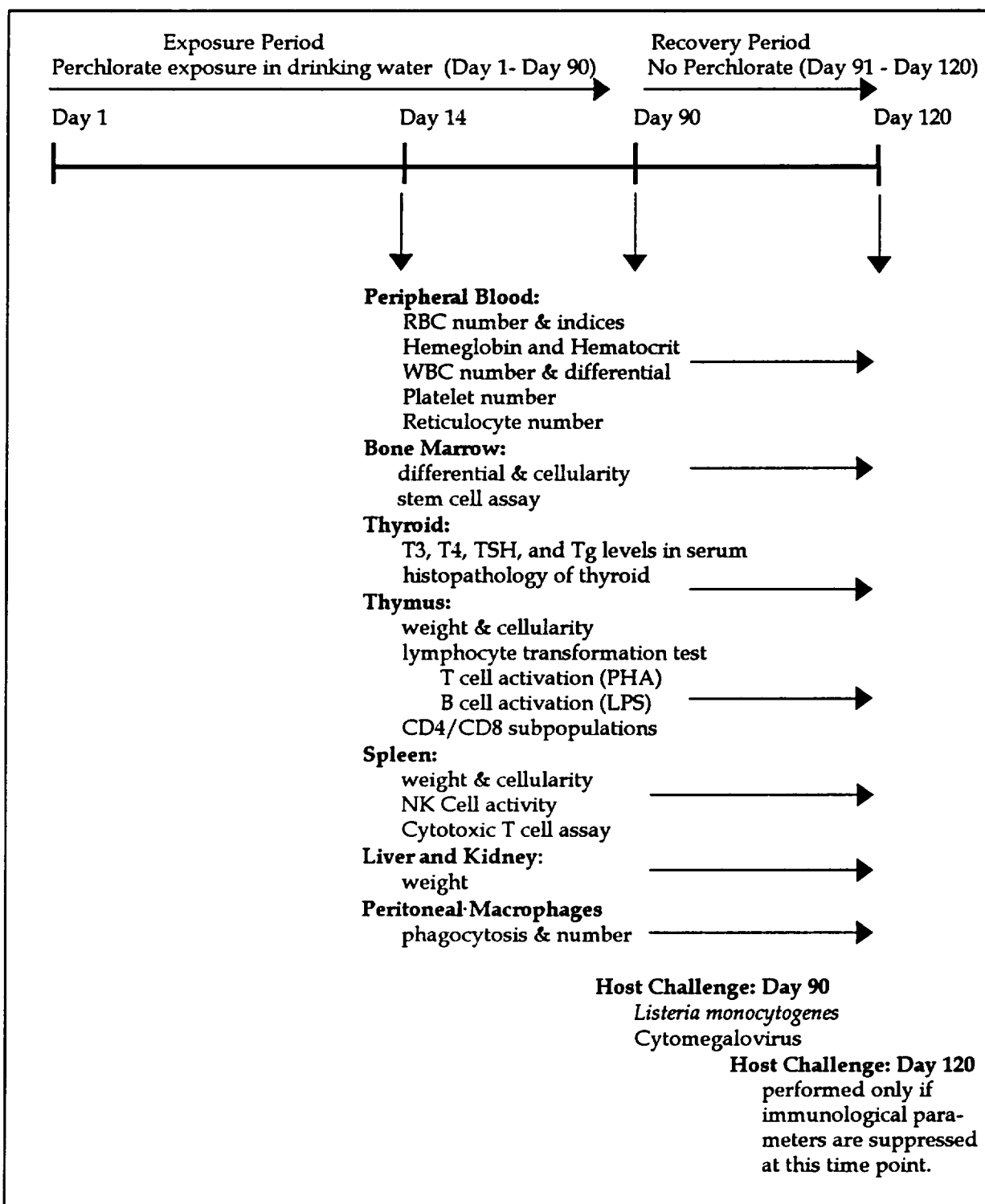
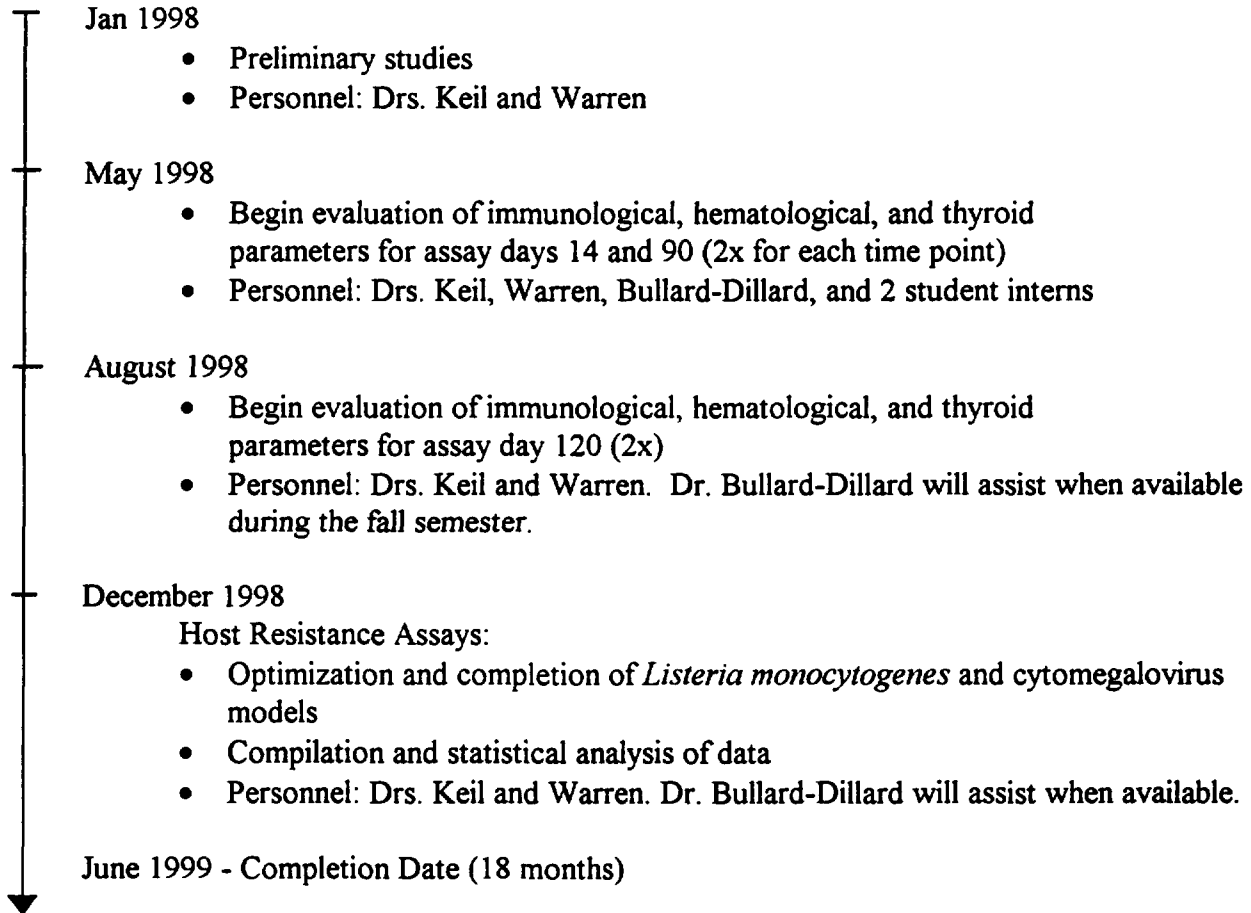


Figure 1. Summary of the Proposed Toxicological Evaluation of Rats Chronically Exposed to Perchlorate in Drinking Water

Time Line for Conducting Proposed Research



Statement of Work

Research tasks will be shared by 3 Ph.D. investigators and 2 senior biology student summer interns.

Drs. Keil and Warren will share the following responsibilities: perform preliminary studies to optimize immunological, hematological, and thyroid assays; determine appropriate AP dose levels; conduct host resistance evaluation of AP in rats; statistically analyze data; interpret results; prepare reports and manuscripts; supervise and instruct the senior student interns.

Dr. Bullard-Dillard will assist in immunological and host resistance assessment of the rats exposed to AP. She will be available primarily during the summer of 1998. Her participation will be limited during the fall and spring semester due to her teaching responsibilities. Thus, the experiments during this time will be carefully scheduled to accommodate Dr. Bullard-Dillard who will participate on a part-time basis.

Senior student interns will assist in thyroid, immunotoxicological, and hematological experiments at MUSC for two months during the summer of 1998. They will also prepare a senior research thesis on an aspect of this study.

Research Affiliation with Claflin College, Orangeburg, South Carolina

Claflin College, an historically African-American college, was founded in 1869 in Orangeburg, South Carolina. Current enrollment is approximately 750 students. Orangeburg is a small city, about 1.5 hours from Charleston, in one of the designated rural areas of South Carolina. In 1993, our university initiated ties with Claflin College by enacting a dual degree program to encourage and support a greater number of African-American applicants to the health professions programs offered by the Medical University. This grant will help strengthen ties with Claflin college by facilitating inter-institutional collaborative research and provide unique, educational, research opportunities for two of their senior students.

Dr. Bullard-Dillard, Assistant Professor in the Department of Biology at Claflin College, will contribute to this project by sharing her expertise in immunology and assisting in experiments. This collaboration will facilitate an opportunity for Dr. Bullard-Dillard to acquire a greater repertoire of immunological and hematological methods, and the means to actively participate in animal research, an opportunity not available at Claflin College. Dr. Bullard-Dillard is also responsible for mentoring several senior biology student theses, and this study will provide at least 2 senior students with an unique research opportunity.

Two senior biology students from Claflin College will be selected to participate in this research facilitating completion of a senior thesis, a requirement of all graduating biology students. At MUSC, these students will acquire skills in laboratory techniques, organization and preparation of experiments, interpretation of data, animal handling, tissue culture methods, and immunological and hematological assays. This opportunity will be most advantageous for Claflin College senior students who will gain unique research experience otherwise unavailable, and who may possibly pursue post-graduate degrees at MUSC.

Proposed Budget**Experimental Costs:**

| | | |
|--------------------|---|-----------------|
| Animals: | Sprague-Dawley rats (purchase and per diem expenses) | \$35,000 |
| Thyroid Histology: | MUSC Histology Core Laboratory Facility (includes slide preparation, staining, and histological evaluation of rat thyroids) | \$ 8,200 |
| Supplies: | flow cytometry center (\$75/hour for 15 hours) | \$ 1,125 |
| | monoclonal fluorescent antibodies (flow cytometry) | \$ 2,000 |
| | tissue culture supplies | \$11,000 |
| | other disposable supplies | \$14,000 |
| | ⁵¹ Cr and ³ H purchase costs and radioactive disposal | \$ 4,000 |
| | T3, T4, and TSH kits- RIA (Diagnostic Products Corp.) | \$ 1,000 |
| Equipment: | Packard Spectracount: 96-well microplate spectrophotometer (includes 6% sales tax) | <u>\$ 6,100</u> |

Subtotal for Experimental Costs: \$ 82,425**Personnel Costs**

| | | |
|-----------------------|---|-----------------|
| Primary Investigator: | Dr. Deborah Keil | |
| | • 30% effort for 18 months | \$21,150 |
| | • benefits (18.987% + hospitalization and insurance) | \$ 4,948 |
| Co-Researcher: | Dr. Alan Warren | |
| | • 100% effort for 18 months (temporary grant employee) | \$60,000 |
| | • benefits (18.987% + hospitalization and insurance) | \$14,500 |
| Co-Researcher: | Dr. Rebecca Bullard-Dillard, <i>Claflin College</i> | |
| | • consultant fees | \$10,000 |
| | • travel expenses to MUSC | \$ 500 |
| Senior Student #1: | student intern for summer, <i>Claflin College</i> | |
| | • wages \$7/hour for 2 months | \$ 2,427 |
| | • benefits (1.1%) | \$ 27 |
| | • temporary relocation expenses to MUSC | \$ 1,500 |
| Senior Student #2: | student intern for summer, <i>Claflin College</i> | |
| | • wages \$7/hour for 2 months | \$ 2,427 |
| | • benefits (1.1%) | \$ 27 |
| | • temporary relocation expenses to MUSC | <u>\$ 1,500</u> |

Subtotal of Personnel Costs: \$119,006

Miscellaneous Costs

| | | |
|---------------|---|----------|
| Travel: | • student travel to regional or national meeting for presentation of research | \$ 2,500 |
| | • travel for 3 researchers to regional or national meeting for presentation of research | \$ 4,500 |
| Publications: | • manuscript publication expenses | \$ 500 |

Subtotal of Miscellaneous Costs: \$ 7,500

Indirect Costs

Indirect Costs (44% minus equipment) = \$ 88,585

Proposed Total Cost

Direct Costs = \$208,931

Indirect Costs = \$ 89,246

Total Cost = \$298,177

Key Personnel:

Deborah E. Keil, Ph.D., MT(ASCP) - Dr. Keil earned her Bachelor of Science Degree in Clinical Laboratory Science at Western Carolina University, North Carolina, in 1991. Subsequently, she was awarded a doctoral fellowship from Mississippi State University and completed her Ph.D. in the Department of Biological Sciences in 1996. Dr. Keil studied immunotoxicology under the tutelage of Dr. Stephen B. Pruett, and her research included comprehensive evaluations of the immunotoxicity of pesticides and therapeutic drugs. Dr. Keil is currently developing novel statistical approaches to quantitative modeling of immunotoxicology data in an effort to improve the assessment of risks from chemical and drug exposures. She is currently an Assistant Professor in the Department of Medical Laboratory Science at the Medical University of South Carolina where she teaches immunohematology and laboratory management, and is establishing a productive research program.

D. Alan Warren, M.P.H., Ph.D. - Dr. Warren received a Bachelor of Science Degree in Environmental Health from the University of Georgia in 1985 and a Master of Public Health Degree from Yale University in 1987. After being employed as an industrial hygienist at the Georgia Tech Research Institute, he returned to the University of Georgia in 1990 to continue his graduate studies. As a doctoral student researching the pharmacokinetics of volatile organic solvents and their neurobehavioral toxicity, he was awarded a Department of Defense Science and Engineering Graduate Fellowship. Upon receiving his Ph.D. Degree in 1995, he joined the Toxicology Division at Wright-Patterson Air Force Base as a National Research Council Research Associate. At Wright-Patterson Air Force Base, he conducts research on the maternal-fetal pharmacokinetics of the prototypical teratogen retinoic acid and its ability to induce limb malformations and cleft palate in the developing mouse. Dr. Warren is also involved in research to examine the cardiac teratogenicity of trichloroethylene and trichloroacetic acid. He is well into the second year of his Research Associateship, after which he hopes to obtain a research faculty appointment.

Rebecca Bullard-Dillard, Ph.D. - Dr. Bullard-Dillard was granted a Bachelor of Science Degree in Biochemistry by North Carolina State University in 1990 and a Ph.D. in Chemistry by the University of South Carolina in 1996. Dr. Bullard-Dillard's doctoral studies included the determination of tissue sites of uptake of radiolabeled retinol-binding protein in the rat as well as sites of uptake and degradation of radiolabeled buckminsterfullerenes in rats and in human keratinocyte tissue cultures. Prior to her doctoral studies, she conducted studies which determined the presence of autoimmune autoantigens in dietary plants and developed cDNA clones capable of producing fibronectin RNA as a means to assess the role of this extracellular matrix protein in heart development. Dr. Bullard-Dillard currently holds the position of Assistant Professor at Claflin College in Orangeburg, South Carolina where she teaches genetics, cell and molecular biology, and a biotechniques laboratory course. Dr. Bullard-Dillard is currently investigating the role of oral tolerance mechanisms in systemic lupus erythematosus disease etiology, and is working to fully establish her research career.

References

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approved by DLAR
D. Keil

December 15, 1997

To: Vicki McGillivray and the MUSC Institutional Animal Care & Use Committee
From: Dr. Deborah Keil
RE: Request for Amendment Approval

I would like to request three changes and an additional immunological endpoint for incorporation in the grant entitled "Effects of Ammonium Perchlorate on Thyroid Hormone Levels, Hematopoiesis, and Immune Status of Sprague-Dawley Rats". These changes are expected to provide a more comprehensive evaluation of the immunological effects of ammonium perchlorate by the substitution and addition of better-suited immunotoxicology assays to be performed in a more appropriate animal model using B6C3F1 mice rather than Sprague-Dawley rats.

#1. Request a modification from the proposed Sprague-Dawley rat model to the B6C3F1 mouse model.

Originally, evaluation of thyroid hormone levels, hematological, and immunotoxicological effects of ammonium perchlorate (AP) was proposed using Sprague-Dawley rats. I am requesting to change from the rat model to the B6C3F1 mouse. This was concluded through extensive consideration and planning for this study, combined with a recent consultation with Dr. M. Selgrade, Chief, Immunotoxicology Branch for the EPA.

There will be several advantages to using the B6C3F1 mouse model in assessing the immunotoxicological effects of AP. As some immunotoxicological assays have been validated in rats, more immunotoxicological assays have been validated in B6C3F1 mice. Evaluation of immune effects could be more comprehensive to include a hypersensitivity assay and some autoimmune assays that have been previously validated in mice (see requested change #2 & #4). Additionally, the *Listeria monocytogenes* challenge model is more routinely used and better-suited for the mouse model rather than the rat model. Furthermore, studies evaluating thyroid, immunological, and hematological effects of AP have not been previously done in mice. The use of mice in this study will provide information regarding the effects of ammonium perchlorate in an alternate species other than the rat. In addition, any changes that may be observed in B6C3F1 mice exposed to AP could be compared to many other B6C3F1 mouse immunotoxicology studies previously compiled in the National Toxicology Program (NTP) database.

Furthermore, the proposed hematological assays and evaluation of thyroid hormone levels have not been modified and will be performed as outlined in the original proposal, except in mice. This should not be problematic because these parameters are routinely measured in mice as in rats.

#2. Request a substitution of the lymphocyte transformation assay to a delayed-type hypersensitivity assay.

Lymphocyte activation assays have been substituted with a delayed type hypersensitivity assay. Lymphocyte activation in the thymus or spleen will not add substantial information regarding immune function when incorporated in the proposed battery of immunological assays and host resistance models (communication with Dr. Selgrade). However, a delayed-type hypersensitivity assay would provide additional information regarding cell-mediated immune function not previously described in the original proposal. Investigating potential hypersensitivity after exposure to AP is particularly relevant. Perchlorate salts have been used to treat hyperthyroidism in Graves' disease patients and some of the described hypersensitivities in these patients may be attributable to the effects of T₄ depletion upon the immune system, particularly inhibition of suppressor T cells (Environmental Resources Management, Inc., 1995). Thus, the addition of the delayed type hypersensitivity assay would be valuable in describing potential hypersensitivity effects of AP.

#3. Request a substitution of the cytomegalovirus (CMV) host challenge model to a B16F10 tumor challenge model.

The CMV model will be replaced with a B16F10 melanoma tumor challenge model. There are several advantages in using the tumor model. In immunotoxicology studies, the tumor model has been used more routinely and consistently whereas the CMV model is difficult to perform and highly variable between laboratories (communication with Dr. Selgrade). The tumor model will also provide information not otherwise known regarding resistance to cancer cell challenge after exposure to AP. Although the infectious challenge agents are different for both models, *in vivo* cooperation of similar immunological parameters are assessed because resistance to CMV or B16F10 tumor challenge depends largely on the function of NK and CTL cells.

Several effector mechanisms are involved in resistance to tumor cells, and the B16F10 tumor model will provide important information as to the status of immunological resistance to cancer cells after exposure to AP. B16F10 tumor cells challenge innate portions of the immune system including macrophages and NK cells as well as specific CTL responses. Early stages of B16F10 tumor resistance require production on interferon and activation of NK cytotoxicity, followed by macrophage antigen presentation and T-cell lymphokine production. Protection from B16F10 cells correlates well with increased NK cell cytotoxicity, followed by latent developing specific cytolytic T-cell response (Markovic and Murasko, 1991). Macrophages are also effective in that they may be fully activated and consequently toxic to cancer cells (Pace and Russell, 1981; Weinberg *et al.*, 1978). The mechanism of resistance to B16F10 cancer cells in mice seem to be relevant to resistance to cancer in humans as indicated by the protective effect of MHC class I-restricted CTLs against tumors in specific human carcinomas. Thus, the B16F10 tumor model will

provide information regarding the *in vivo* interaction of several immunological mechanisms and will be a meaningful model to incorporate in this study.

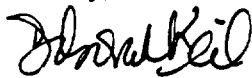
#4. Request the addition of autoimmune evaluation.

Incorporation of autoimmune endpoints in this study would be useful for it is not known if exposure to AP may affect the development of autoimmune disease. It would be relatively easy to obtain extra mouse serum to determine the presence of antinuclear antibodies (ANA). Dr. Rebecca Bullard-Dillard of Claflin College has previous experience in detecting autoantibodies in mice and will assist in performing these ELISAs.

Summary

The immunological parameters and host resistance models selected for use in the present study were designed to provide a comprehensive indication of the status of the immune system after exposure to ammonium perchlorate. With the use of the B6C3F1 mouse model rather than the rat model, more validated immunological assays may be utilized resulting in an improved assessment of immunological effects. Hematological parameters and thyroid levels will be assessed as originally proposed, except in mice. I appreciate your consideration of these grant modifications and would welcome any comments or concerns regarding the proposed changes. Enclosed is the revised version of the animal protocols incorporating the changes described here.

Sincerely,



Deborah E. Keil, PhD
Assistant Professor
Dept. of Medical Laboratory Sciences
Medical University of South Carolina

Revised: Effects of Ammonium Perchlorate on Thyroid Hormone Levels, Hematopoiesis, and Immune Status of *B6C3F1 Female Mice*

The hypotheses to be tested are:

1. **Low levels of ammonium perchlorate will dose-responsively cause changes in thyroid, hematological, and immunological parameters after an exposure administered daily for 14 or 90 days in the drinking water fed to B6C3F1 female mice.**
2. **Correspondingly, low levels of ammonium perchlorate administered for 90 days in the drinking water will dose-responsively alter susceptibility to disease in B6C3F1 female mice challenged with tumor cells or infectious bacterial agent.**
3. **After a 90-day exposure to low levels of ammonium perchlorate in the drinking water of B6C3F1 female mice, any changes in the thyroid, hematological, or immunological functions will be restored to normal in 30 days after cessation of ammonium perchlorate.**

Proposed Use of the *B6C3F1 Female Mice*

The following is a time line indicating the proposed assays for thyroid, hematological, and immunotoxicology evaluation AP in B6C3F1 female mice. Several assays will be measured at 3 different time points to identify the effects of AP after 14 and 90 days of exposure, and after a subsequent 30 day recovery period.

In efforts to use the least number of experimental B6C3F1 female mice, several assays will be measured from each animal through the coordinated efforts of Dr. Warren, Dr. Keil, and trained graduate students. Dr. Warren and Dr. Keil have had extensive experience and success in coordinating such a battery of experiments for several animals.

Thyroid, Hematological, and Immunological Parameters (revised = *italics*)

Thyroid assessment:

Serum- quantitation of T₃, T₄, Thyroid Stimulating Hormone (TSH)

Thyroid- histopathology

Immunological parameters:

Thymus- weight and cellularity, CD4/8 subpopulations

Spleen- weight and cellularity, natural killer cell activity

Cytotoxic T cell activity

Delayed-type hypersensitivity assay

Peritoneal macrophages- phagocytosis

Serum – anti-nuclear antibodies by ELISA

Hematological parameters:

Peripheral blood- RBC number and indices, hemoglobin and hematocrit, WBC number & differential, platelet number, reticulocyte number

Liver and Kidney- weight

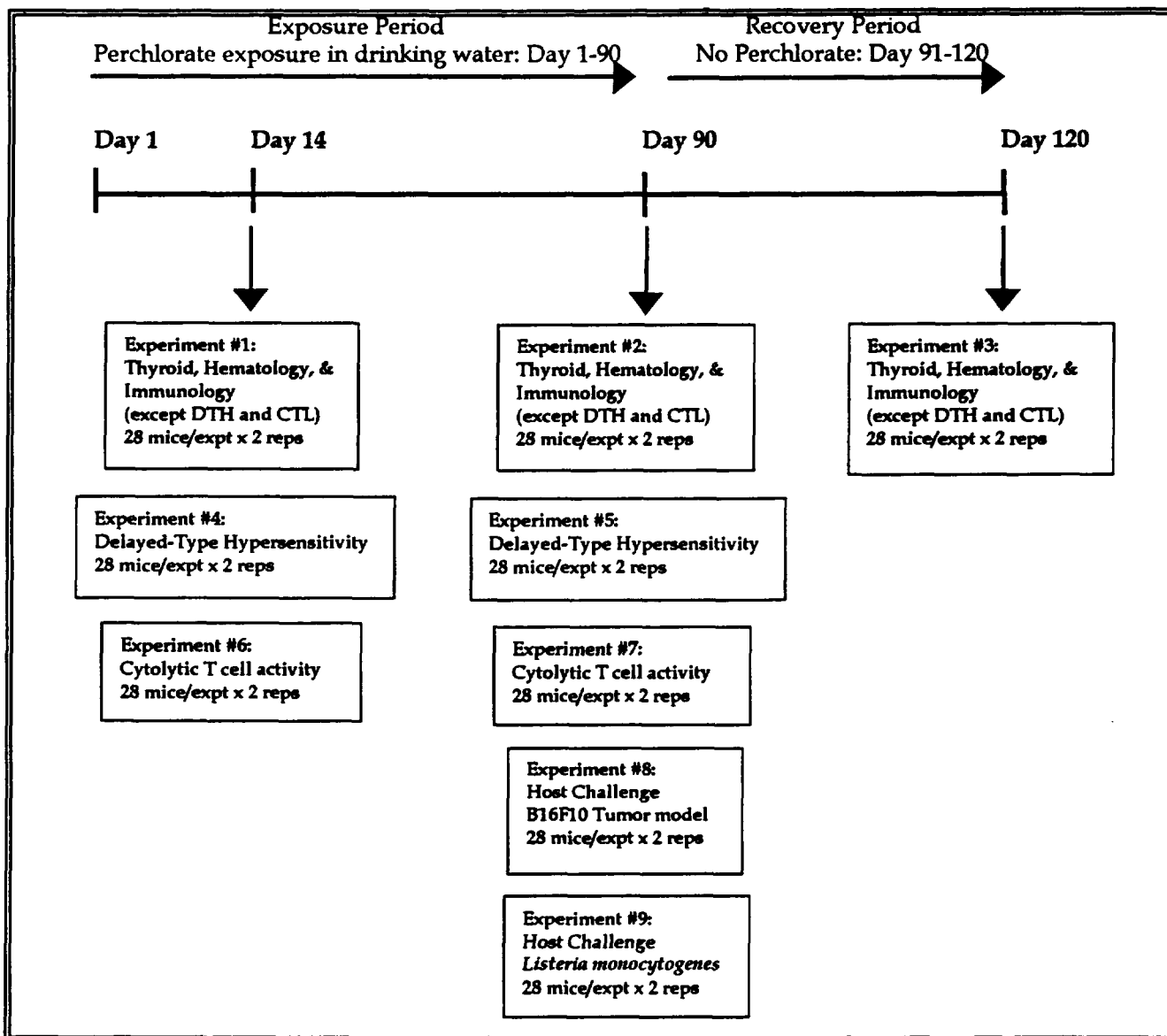


Figure 1. Time line indicating experiments using B6C3F1 female mice for thyroid, immunological, hematological, and host challenge assessment after subchronic, low-level exposures to perchlorate in drinking water.

Host Challenge Models

Resistance to Listeria monocytogenes Challenge

This host challenge model is recommended in the immunotoxicology tier approach (Luster, 1988; Luster, 1992) because it effectively evaluates immunological resistance that is dependent on the *in vivo* cooperation of several parameters such as T cells, natural killer cell, macrophages, and neutrophils (Baldrige, 1990; Vos, 1977, Czuprynski, 1994; Dunn, 1991). Challenge with *Listeria monocytogenes* in mice requires an intravenous injection in

the range of approximately 1×10^4 total bacteria (approximately an LD₂₀). Death will not be used as an endpoint. Alternatively, *mice* will be euthanized with carbon dioxide before any apparent suffering on day 4 after immunization with *Listeria monocytogenes*. To obtain blood, mice will anesthetized with metophane and bled retroorbitally. Immediately following this procedure and before the mouse obtains consciousness, mice will be euthanized with compressed carbon dioxide from a cylinder in a specially designed gas chamber. Bacterial colony counts will be performed on the liver, blood and spleen to evaluate immunological resistance to *Listeria monocytogenes* challenge during a low-level exposure to AP.

Delayed-type Hypersensitivity Assay

This assay is similar to the host resistance assay in that it requires an intravenous sublethal challenge with *Listeria monocytogenes* (approximately 1000-3000 CFU) in mice exposed to AP for 14 or 90 days. Mice will be euthanized (as discussed in the following section) 7 days following the challenge. Spleens will be removed and exposed to heat-killed *Listeria monocytogenes*. Essentially, the cells will be pulsed with ³H, harvested, and quantitated.

Ammonium Perchlorate Exposure

The perchlorate anion is a uniquely stable, high oxygen density grouping and exists as a colorless, orthorhombic crystals with the following properties:

| | |
|----------------------|----------------------------------|
| CAS registry number | 7790-98-9 |
| Chemical formula | NH ₄ ClO ₄ |
| thanMolecular weight | 117.49 g/mol |
| Density | 1.95 g/cm ³ at 25°C |
| Solubility | 20 g 100cm ³ at 20°C |

Preliminary Range-Finding Studies for AP Exposures

A minimal number of mice (total = 50) will be needed to establish appropriate perchlorate dose levels in preliminary range-finding studies. To minimize the number of mice for this study, reference will be made to two previous studies that evaluated thyroid toxicity and changes in body weight in Sprague-Dawley rats exposed to AP in drinking water (Caldwell, *et al.*, 1996; Mannisto, *et al.*, 1970). The levels of perchlorate desired will be relatively low doses that will not cause overt toxicity nor decrease the body weight more than 10% for the duration of the 14 or 90-day exposure. Thyroid, hematological, and immunological parameters will also be measured in mice used for preliminary range finding studies. This will permit appropriate selection of doses concerning changes in the parameters and allow for optimization of assays. If, during the range-finding studies overt toxicity is detected after AP exposure, the mice will be euthanized. Overt toxicity will be defined as a rapid weight loss of greater than 10%, and may include signs such as diarrhea, alopecia, wheezing, central nervous system abnormalities, lack of grooming, and any other clinical signs judged by an experienced veterinarian employed by the DLAR facility.

Use of B6C3F1 female mice

This study requires living animals with the capacity to both metabolize and respond to the physiological effects of AP. Alternative techniques such as tissue culture or computer simulations were considered but determined to be inadequate for the purposes of this research.

During the experiments, female B6C3F1 mice (viral antibody free) will be housed individually per cage at an ambient temperature of 22C, relative humidity of 55±5%, and with a 12 hour light-dark cycle in the DLAR facility. AP will be administered via drinking water in the 3 treatment groups (i.e. low, medium & high exposure to AP), and water only will be available for the control group. The volumes of water consumed will be monitored. They will be fed standard mouse chow ad libidum. Exposure to AP in the drinking water will begin when the mice are 8-9 weeks old. This level of maturity will ensure a stable thyroid metabolism and minimize variability in endpoints. Body weight will be monitored daily for the duration of the experiments, and the doses of AP to be used will not cause more than a 10% decrease in body weight, nor any other signs of overt toxicity.

Differences in the gender response to AP will not be considered in this preliminary study. Therefore, female B6C3F1 mice and not male mice will be used. B6C3F1 female mice are the standard in immunotoxicology evaluations and used to compile the National Toxicology Database.

Total Number of Mice Required

Three doses of AP in the drinking water, and a control group administered drinking water without AP, will be included in each experiment. In addition, 7 mice/dose of AP will be used for a total of 28 mice per experiment. Mice will be housed individually in the DLAR facility and in accordance to DLAR guidelines.

7 mice x 4 treatments (3 doses of AP and control) = 28 mice/experiment
28 total mice/expt x 9 expts x 2 replications of each expt = 504 total mice

Preliminary range finding studies = 50 B6C3F1 female mice

| | |
|-----------------------------------|---|
| Total number of mice for proposal | = range finding studies + experiments |
| | = 50 + 504 |
| | = 554 total mice to be used in this study |

Statistics

Mice will be randomly assigned to each treatment group. The unit of comparison will be the individual mouse. Analysis of variance will be performed using Dunnett's *t* test with a significance level of $p < 0.05$. Seven animals per group will permit detection of significant changes within treatments and allow for possible high variation within treatment groups.

Veterinary Care of the Mice

“Animals will be housed in the facilities for laboratory animals provided by the Division of Laboratory Animal Resources under the direction of M. Michael Swindle, D.V.M., a diplomate of ACLAM. An assurance statement is on file with OPRR/DHHS detailing program for laboratory animal care of MUSC. MUSC has ongoing full accreditation from AAALAC effective November 5, 1987.”

Procedures and Euthanasia Performed on B6C3F1 female mice

Perchlorate Exposure Via Drinking Water:

This is an appropriate route of exposure for this study because humans would also be exposed via drinking water from contaminated groundwater wells.

Known quantities of AP will be added to defined volumes of drinking water. Exact quantities of AP added to drinking water will be determined during preliminary experiments. The water will be available ad libitum and the volume of water consumed by each individually housed mouse will be monitored.

AP is a salt and is stable in the drinking water. Thus, AP would not be toxic to persons in contact with the animal room or animals. However, the appropriate precautions for the disposal of excess AP contaminated water will be performed as per OSHA guidelines.

The taste of the AP salt in drinking water is not expected to influence water intake by the mice. Two independent studies (Mannisto, 1970 and Caldwell, 1996) exposed Sprague-Dawley rats via drinking water at concentrations ranging from 1.25 to 500 mg/L, and there was no indication that water intake was affected. However, we intend to monitor mice for such an effect by comparing the volume intake of water per animal as well as recording body weights.

The levels of perchlorate desired will be relatively low doses that will not cause overt toxicity nor decrease the body weight more than 10% for the duration of the 14 or 90-day exposure. If, during the range-finding studies overt toxicity is detected after AP exposure, the mice will be immediately euthanized. Overt toxicity will be defined as a rapid weight loss of greater than 10%, and may include other signs such as diarrhea, alopecia, wheezing, central nervous system abnormalities, lack of grooming (Browder, E. J., “Death as an endpoint”), and any other clinical signs judged by an experienced veterinarian employed by the DLAR facility.

Intravenous Injection:

In the bacterial host challenge model and the delayed type hypersensitivity assay, mice will be challenged a single time on day 14 or 90 with *Listeria monocytogenes* by way of an intravenous injection in the tail vein. Intravenous injections will be aseptically performed with a 25 gauge needle, sterile syringe, and approximately 0.2 mL in volume of sterile saline used as the suspension for *Listeria monocytogenes*. The mouse will be restrained in

an appropriate restraining device (acrylic tube) for no longer than 2 minutes. A warming device (used routinely in hospitals for baby heel sticks) will be applied to the tail while the animal is restrained. This will increase circulation of blood to the tail and visibility of the veins for a successful intravenous injection. Mice will not be anesthetized for this procedure since this will only cause minimal discomfort to the animal. This investigator is both skillful and quick in performing tail vein injections. Although no complications are expected, either Drs. Keil or Warren will observe mice for at least 10 minutes after injection.

Peritoneal Injection:

In the tumor host challenge model and the cytolytic T cell assay, mice will be challenged a single time on day 14 or 90 with either B16F10 melanoma tumors or P815 tumors by way of a peritoneal injection. Peritoneal injections will be aseptically performed with a 25 gauge needle and sterile syringe, approximately 0.2 ml in volume, and sterile saline used as the suspension for B16F10 tumor cells. The mouse will be appropriately and comfortably handheld by this investigator while exposing the peritoneum for injection. Mice will not be anesthetized for this procedure since this will only cause minimal discomfort to the animal. This investigator is both skillful and quick in performing peritoneal injections. Although no complications are expected, either Drs. Keil or Warren will observe mice for at least 10 minutes after injection. Mice will be euthanized within 14 days post tumor challenge to prevent the animal from experiencing discomfort or pain from bearing large tumor burdens.

Bleeding of Mice:

Mice will be anesthetized in a chamber with methoxyflurane until the mouse is breathing comfortably and no longer conscious. A retroorbital bleed will be performed on the mouse to obtain a maximum amount of 1 ml of whole blood required for immunotoxicological assays. A heparinized capillary tube will be gently placed behind the mouse eye to access a bed of capillaries rich in blood flow. After bleeding and prior to consciousness of the mouse, the mouse will be immediately euthanized with carbon dioxide asphyxiation. Euthanasia immediately following the bleeding procedure will limit the discomfort and distress to the animal. Although no complications are expected, mice will be observed and euthanized at any sign of unusual distress or discomfort experienced by the animal during this procedure.

Euthanasia:

Mice will be euthanized on day 14, 90 or 120 with compressed carbon dioxide (60%) from a cylinder in a specially designed gas chamber for a period of 5 minutes to ensure the death of the mouse. This will be performed in a separate room from live animals. This is an acceptable method of euthanasia for mice (JAVMA, 1993, 202:236), and is not known to adversely interfere with any of the proposed experiments.

Alternatives to Proposed Mouse Procedures

Alternatives to Tail Vein Intravenous Injection:

Injection in the tail vein is the standard method used to reliably challenge the mouse intravenously. This will not cause more than momentary, slight pain or distress to the mouse. Mice will be carefully restrained in an approved restrainer resembling an acrylic tube to minimize discomfort and stabilize the animal for a non-problematic injection. Dr. Warren and Dr. Keil are experienced at intravenous injections via tail veins, and this will expedite handling time of each mouse. For the purposes of the host resistance challenges, a medline database search did not reveal an alternative method to intravenous injection via the tail vein.

Alternatives to Peritoneal injection:

A peritoneal injection is a common method used to immunize mice and this is the most appropriate method to challenge mice with B16F10 melanoma tumor cells. Mice will not be anesthetized for this procedure since this will only cause minimal discomfort and distress to the animal. This investigator is both skillful and quick in performing peritoneal injections.

Alternative Methods of Bleeding Mice:

It is possible to obtain blood from a cardiac puncture or from the vena cava of mice that have been euthanized with carbon dioxide. However, it has been my experience that the blood clots quickly, and only small, inadequate volumes of whole blood can be obtained (less than 1 ml). Retroorbital bleeding is an efficient and reliable method to obtain larger volumes of blood. Since the mouse is anesthetized with methoxyflurane during this procedure and will be euthanized with carbon dioxide prior to regaining consciousness, little discomfort and distress will be experienced by the mice.

Euthanasia:

Mice will be euthanized in an acceptable manner with carbon dioxide (JAVMA, 1993, 202:236. This should cause only momentary or slight pain to the animal and is not known to interfere with the assays suggested in this proposed study.

Literature Search and References

The following databases, Toxline and Medline, were accessed to assure that no other studies of this kind have been previously reported. Research related to this study or cited in this proposal have been listed here.

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December 1, 1997.

Request for Modification of Grant #DSWA 01-97-1-0008

Grant Title: Effects of Ammonium Perchlorate on Thyroid Hormone Levels,
Hematopoiesis, and Immune Status of Sprague-Dawley Rats

Submitted to:
Major Steven Huff
Technical Correspondent
Defense Special Weapons Agency
6801 Telegraph Road
Alexandria, VA 22310-3398

Major Huff,

I would like to request three changes and an additional immunological endpoint for incorporation in the grant entitled "Effects of Ammonium Perchlorate on Thyroid Hormone Levels, Hematopoiesis, and Immune Status of Sprague-Dawley Rats". These changes are expected to provide a more comprehensive evaluation of the immunological effects of ammonium perchlorate by the substitution and addition of better-suited immunotoxicology assays to be performed in a more appropriate animal model using B6C3F1 mice rather than Sprague-Dawley rats. The current research budget is expected to accommodate the following changes and additional funds are not requested. The completion date for this study will also remain the same.

#1. Request a modification from the proposed Sprague-Dawley rat model to the B6C3F1 mouse model.

Originally, evaluation of thyroid hormone levels, hematological, and immunotoxicological effects of ammonium perchlorate (AP) was proposed using Sprague-Dawley rats. I am requesting to change from the rat model to the B6C3F1 mouse. This was concluded through extensive consideration and planning for this study, combined with a recent consultation from Dr. M. Selgrade, Chief, Immunotoxicology Branch for the EPA.

There will be several advantages to using the B6C3F1 mouse model in assessing the immunotoxicological effects of AP. As some immunotoxicological assays have been validated in rats, more immunotoxicological assays have been validated in B6C3F1 mice.

Evaluation of immune effects could be more comprehensive to include a hypersensitivity assay and some autoimmune assays that have been previously validated in mice (see requested change #2 & #4). Additionally, the *Listeria monocytogenes* challenge model is more routinely used and better-suited for the mouse model rather than the rat model. Furthermore, studies evaluating thyroid, immunological, and hematological effects of AP have not been previously done in mice. The use of mice in this study will provide information regarding the effects of ammonium perchlorate in an alternate species other than the rat. In addition, any changes that may be observed in B6C3F1 mice exposed to AP could be compared to many other B6C3F1 mouse immunotoxicology studies previously compiled in the National Toxicology Program (NTP) database.

Furthermore, the proposed hematological assays and evaluation of thyroid hormone levels have not been modified and will be performed as outlined in the original proposal, except in mice. This should not be problematic because these parameters are routinely measured in mice as in rats.

#2. Request a substitution of the lymphocyte transformation assay to a delayed-type hypersensitivity assay.

Lymphocyte activation assays have been substituted with a delayed type hypersensitivity assay. Lymphocyte activation in the thymus or spleen will not add substantial information regarding immune function when incorporated in the proposed battery of immunological assays and host resistance models (communication with Dr. Selgrade). However, a delayed-type hypersensitivity assay would provide additional information regarding cell-mediated immune function not previously described in the original proposal. Investigating potential hypersensitivity after exposure to AP is particularly relevant. Perchlorate salts have been used to treat hyperthyroidism in Graves' disease patients and some of the described hypersensitivities in these patients may be attributable to the effects of T₄ depletion upon the immune system, particularly inhibition of suppressor T cells (Environmental Resources Management, Inc., 1995). Thus, the addition of the delayed type hypersensitivity assay would be valuable in describing potential hypersensitivity effects of AP.

#3. Request a substitution of the cytomegalovirus (CMV) host challenge model to a B16F10 tumor challenge model.

The CMV model will be replaced with a B16F10 melanoma tumor challenge model. There are several advantages in using the tumor model. In immunotoxicology studies, the tumor model has been used more routinely and consistently whereas the CMV model is difficult to perform and highly variable between laboratories (communication with Dr. Selgrade). The tumor model will also provide information not otherwise known regarding resistance to cancer cell challenge after exposure to AP. Although the infectious challenge agents are different for both models, *in vivo* cooperation of similar immunological parameters are assessed because resistance to CMV or B16F10 tumor challenge depends largely on the function of NK and CTL cells.

Several effector mechanisms are involved in resistance to tumor cells, and the B16F10 tumor model will provide important information as to the status of immunological resistance to cancer cells after exposure to AP. B16F10 tumor cells challenge innate portions of the immune system including macrophages and NK cells as well as specific CTL responses. Early stages of B16F10 tumor resistance require production on interferon and activation of NK cytotoxicity, followed by macrophage antigen presentation and T-cell lymphokine production. Protection from B16F10 cells correlates well with increased NK cell cytotoxicity, followed by latent developing specific cytolytic T-cell response (Markovic and Murasko, 1991). Macrophages are also effective in that they may be fully activated and consequently toxic to cancer cells (Pace and Russell, 1981; Weinberg *et al.*, 1978). The mechanism of resistance to B16F10 cancer cells in mice seem to be relevant to resistance to cancer in humans as indicated by the protective effect of MHC class I-restricted CTLs against tumors in specific human carcinomas. Thus, the B16F10 tumor model will provide information regarding the *in vivo* interaction of several immunological mechanisms and will be a meaningful model to incorporate in this study.

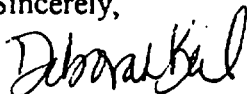
#4. Request the addition of autoimmune evaluation.

Incorporation of autoimmune endpoints in this study would be useful for it is not known if exposure to AP may affect the development of autoimmune disease. It would be relatively easy to obtain extra mouse serum to determine the presence of antinuclear antibodies (ANA). Dr. Rebecca Bullard-Dillard of Claflin College has previous experience in detecting autoantibodies in mice and will perform these assays. No extra research funds are requested.

Summary

The immunological parameters and host resistance models selected for use in the present study were designed to provide a comprehensive indication of the status of the immune system after exposure to ammonium perchlorate (see figure 1 on following page for complete listing of parameters). With the use of the B6C3F1 mouse model rather than the rat model, more validated immunological assays may be utilized resulting in an improved assessment of immunological effects. Hematological parameters and thyroid levels will be assessed as originally proposed, except in mice. Furthermore, no additional research funds are requested for these adjustments in the proposal. I appreciate your consideration of these grant modifications and would welcome any comments or concerns regarding the proposed changes.

Sincerely,



Deborah E. Keil, PhD

Assistant Professor

Dept. of Medical Laboratory Sciences

Medical University of South Carolina

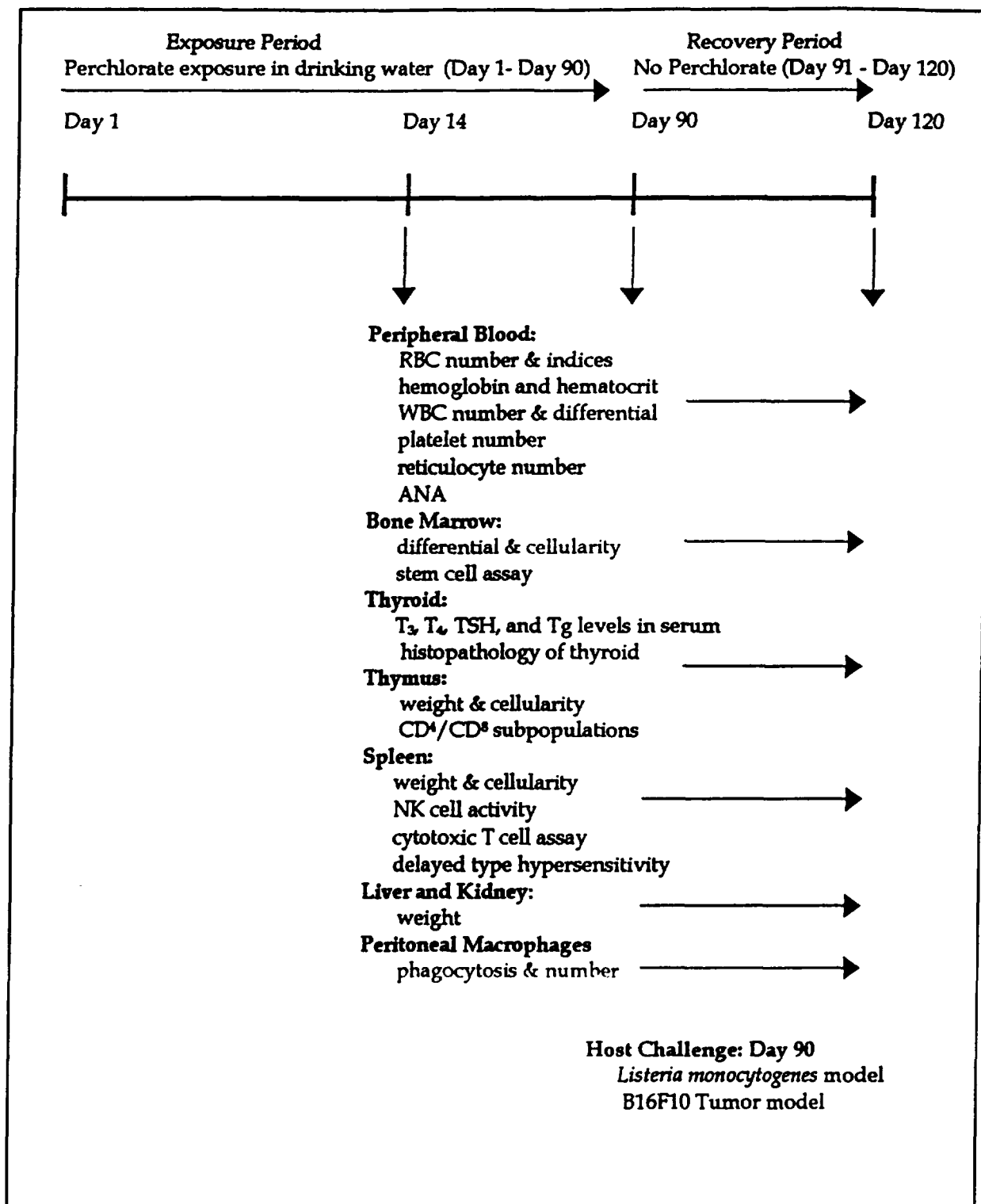


Figure 1. Updated summary of the proposed toxicological evaluation of B6C3F1 mice chronically exposed to perchlorate in drinking water